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14. ABSTRACT

Breast cancer is the most common cancer in women. Tamoxifen has been a front-line treatment for estrogen receptor alpha (ERα)-positive breast tumors in premenopausal women. However resistance to tamoxifen occurs in many patients. ERα still plays a critical role in the growth of breast cancer cells with acquired tamoxifen resistance, suggesting that ERα remains a valid target for treatment of tamoxifen-resistant breast cancer. In an effort to identify novel regulators of ERα signaling, through a small-scale siRNA screen against histone methyl modifiers, we found WHSC1, a histone H3K36 methyltransferase, as a positive regulator for ERα signaling in breast cancer cells. We demonstrated that WHSC1 is recruited to the ERα gene by interacting with the BET protein BRD3/4, and facilitates ERα gene expression. The small-molecule BET protein inhibitor JQ1 potently suppressed the classic ERα signaling pathway and the growth of tamoxifen-resistant breast cancer cells in culture. Through the first year of funding, we have made the following discovery: 1. JQ1 targets both ERα and MYC pathways in Tamoxifen-resistant cells; 2. JQ1 exerts tumor suppression effect mainly through regulating cell cycle genes; 3. JQ1 gene signature correlates with better clinical outcomes; 4. Tamoxifen-resistant MCF7 cells are more sensitive to JQ1 treatment; and GATA3 appear to be one of determinants for JQ1 sensitivity.

15. SUBJECT TERMS
Tamoxifen, bromodomain, resistance

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Table of Contents

Page

Introduction

Breast cancer is the most common cancer in women. Tamoxifen has been a front-line treatment for estrogen receptor alpha (ERα)-positive breast tumors in premenopausal women. However resistance to tamoxifen occurs in many patients. ERα still plays a critical role in the growth of breast cancer cells with acquired tamoxifen resistance, suggesting that ERα remains a valid target for treatment of tamoxifen-resistant breast cancer. In an effort to identify novel regulators of ERα signaling, through a small-scale siRNA screen against histone methyl modifiers, we found WHSC1, a histone H3K36 methyltransferase, as a positive regulator for ERα signaling in breast cancer cells. We demonstrated that WHSC1 is recruited to the ERα gene by interacting with the BET protein BRD3/4, and facilitates ERα gene expression. The small-molecule BET protein inhibitor JQ1 potently suppressed the classic ERα signaling pathway and the growth of tamoxifen-resistant breast cancer cells in culture. It was reported that JQ1 functions mainly by down-regulating MYC transcription[1,2]. My results suggest that JQ1 might be a useful drug in treatment of Tamoxifen resistant breast cancer by shutting down the expression of both ERα and MYC at the transcriptional level. In this grant, I proposed to (1) Determine the molecular mechanisms of JQ1 effect on Tamoxifen-resistant MCF7L breast cancer cells by microarray analysis; (2) Determine the therapeutic effect of JQ1 on a panel of well-characterized endocrine resistant preclinical models; (3) Study JQ1 in vivo anti-tumor function using the Tamoxifen-resistant breast cancer xenograft model in nude mouse.

Body

For the first year, we proposed to determine the molecular mechanisms of JQ1 on Tamoxifen-resistant breast cancer cells by microarray analysis. And the following are the results we obtained:

1. Microarray analysis identifies cell cycle genes as major targets of JQ1

My preliminary results showed that JQ1 represses ERα gene expression. To determine the global signaling pathways that are altered by JQ1 in addition to ER, microarray analysis was performed using Tam-R MCF7 cells treated with vehicle or 0.2 μM of JQ1 (in triplicates). The Affymetrix GeneChip Human Gene 1.0 ST array containing 764,885 distinct probes covering 28,869 well-annotated genes on a single array has been used. When applying a threshold of $\log2 < -0.2$ or $\log2 > 0.2$, we identified 652 down-regulated genes and 219 up-regulated genes in JQ1-treated cells (Fig. 1a). Fig. 1b showed the biological pathways negatively affected by JQ1, and Table 1 lists all the genes up-regulated or down-regulated in major biological pathways by KEGG pathway analysis. Among them, the cell cycle is the primary pathway being affected since cell cycle-related gene expression was significantly altered by JQ1 treatment (Fig. 2). Consistent with this observation, by flow cytometry analysis, we found that Tam-R MCF7 cells were arrested at G1 phase after JQ1 treatment for 24 hrs, while parental cells were arrested at G1 phase after > 48 hrs of JQ1 treatment (Fig. 3).

2. JQ1 targets both ERα and MYC pathways in tamoxifen-resistant breast cancer cells

JQ1 was reported to inhibit MYC signaling in previous studies. In our microarray analysis, many altered cell cycle-regulated genes were MYC target genes, such as E2f1, MCM5, Cdc25A, Cdc25C, CKD6, and Cdc6 (Fig. 2). Thus we determined the effect of JQ1 treatment on the mRNA levels of MYC in parental and Tam-R MCF7 cells. Shown in Fig. 4, MYC was dramatically reduced in JQ1-treated Tam-R MCF7 cells, but only slightly reduced in parental MCF7 cells. This result indicates that MYC is indeed a JQ1 target gene in Tam-R MCF-7 cells, and this partially explain why JQ1 has more potent inhibitory effect on the growth of Tam-R cells than on the parental cells. Our result suggests that JQ1 targets both ERα and MYC pathways in tamoxifen-resistant breast cancer cells.

3. JQ1 gene signature correlates with better clinical outcomes

Next we want to find out how JQ1-regulated gene pathways correlate with clinical outcomes. Therefore, in collaboration with Dr. Chad Creighton at Duncan Cancer Center, we generated a JQ1-regulated gene signature with these JQ1 target genes[3]. Using a compendium of nine separate breast tumor expression array datasets, we scored human breast tumors based on the manifestation of the JQ1 gene signature. In ER-positive tumors (N=682), high manifestation of the JQ1 signature was associated with better patient outcome (Fig. 5, Log-rank P=0.001), while in ER-negative tumors (N=309), no survival association was found. This data further support the functional significance of JQ1 on ER signaling in breast cancer.

4. JQ1 induces apoptosis in Tamoxifen resistant MCF7 cells

After 2 days of JQ1 treatment, Tam-R cells began to die, suggesting that prolonged cell cycle arrest may induce apoptosis. This was confirmed by the appearance of cleaved PARP-1 protein in Tam-R MCF7 cells. In contrast, parental MCF7 cells didn't undergo apoptosis (Fig. 6).

5. GATA3 as one of determinants of JQ1 sensitivity

My preliminary results showed that Tam-R breast cancer cells are more sensitive to JQ1. Related to this observation, we noticed when cells were treated with JQ1 for up to three days, ERα mRNA was persistently suppressed in Tam-R MCF-7

cells. In contrast, in parental MCF-7 cells, ERα mRNA level was abolished initially, but recovered after prolonged treatment (Fig. 4). And MYC mRNA level responded to JQ1 treatment similarly to ERα in parental and Tam-R MCF7 cells. These results demonstrate that ERα and MYC are JQ1 target genes in Tam-R MCF-7 cells, and that sustained suppression of ERα and MYC by JQ1 probably contributes to its more potent anticancer activity on Tam-R breast cancer cells. To gain more mechanistic insight into this observation, we found that GATA3, a key regulator of ER gene expression25, is highly expressed in parental MCF-7 cells, but not in Tam-R cells (Fig. 7a and 7b). And in parental MCF-7 cells, GATA3 expression is further increased by JQ1 treatment (Fig. 7a). When we knocked down GATA3 using siRNA, the parental MCF-7 cells became more sensitive to JQ1 treatment (Fig. 7d). Thus, our results suggest that other key transcription factors, such as GATA3 in parental MCF-7 cells, could have contributed to the JQ1 resistance with prolonged treatment. A decrease in such factors might contribute to epigenomic environmental changes on the ERα promoter, resulting greater JQ1 sensitivity in Tam-resistant lines.

Key research accomplishments

Through the first year of funding, we have made the following conclusions:

- Bromodomain inhibitor JQ1 targets both ERα and MYC pathways in Tamoxifen-resistant cells;
- JQ1 exerts tumor suppression effect mainly through regulating cell cycle genes;
- JQ1 gene signature correlates with better clinical outcomes;
- Tamoxifen-resistant MCF7 cells are more sensitive to JQ1 treatment; and GATA3 appear to be one of determinants for JQ1 sensitivity.

Reportable outcomes:

1. I attended the poster session at the 2014 Keystone (Cancer Epigenetics) meeting, with the title "An epigenomic approach to therapy for Tamoxifen-resistant breast cancer".
2. The microarray results generated from this study have been deposited to Gene Expression Omnibus (GET), with accession number GSE49124.

Conclusion:

The central idea of this study is to apply a novel epigenetic drug, JQ1, to treat endocrine-resistant breast cancer. Previously we have found that JQ1 inhibits the growth of cultured tamoxifen-resistant breast cancer cells by down-regulating ERα gene expression. During the first year of funding, we have performed a series of mechanistic studies, and identified the molecular and cellular pathways targeted by JQ1; and determined that low level of GATA3, a transcriptional regulator of ERα, as one important reason to cause hyper-sensitivity toward JQ1 in tamoxifen-resistant cells. Moreover, through microarray analysis, we generated JQ1 gene signature, and found it correlate with better clinical outcomes in ER-positive breast tumors.

References:

1 Mertz, J. A. *et al.* Targeting MYC dependence in cancer by inhibiting BET bromodomains. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 16669-16674, doi:10.1073/pnas.1108190108 (2011).

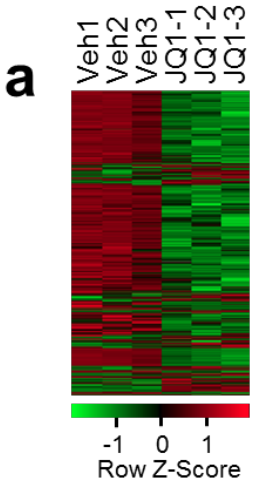
2 Delmore, J. E. *et al.* BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell* **146**, 904-917, doi:10.1016/j.cell.2011.08.017 (2011).

3 Integrated genomic analyses of ovarian carcinoma. *Nature* **474**, 609-615, doi:10.1038/nature10166 (2011).

Appendices:

N/A

Supporting data:
Fig. 1



b

KEGG Pathways	Gene Count	% of genes	P Value
Cell cycle	27	4.19906687	3.36E-14
DNA replication	10	1.55520995	2.22E-06
Pyrimidine metabolism	11	1.71073095	0.0013837
Systemic lupus erythematosus	11	1.71073095	0.00189615
Homologous recombination	6	0.93312597	0.00229482
Purine metabolism	13	2.02177294	0.00582703
Progesterone-mediated oocyte maturation	8	1.24416796	0.02703209
Mismatch repair	4	0.62208398	0.04219004

Fig. 1. (a) Heatmap of expression levels for the genes differentially expressed upon treatment with JQ1. Tam-R MCF7 cells were treated with 0.2 µM of JQ1 or vehicle (DMSO) for 24 hrs before harvest for microarray analysis. (b) Biological pathways were identified by microarray analysis. KEGG pathways were determined by the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov) based on the gene list that is down-regulated by JQ1.

Table 1a, pathways upregulated by JQ1

KEGG pathways	Genes up-regulated in JQ1 treated cells
Systemic lupus erythematous	HIST2H2AA, HIST2H4A/B, HIST1H2AC, HIST1H2BC, HIST1H3H, HIST1H2BD
Nitrogen metabolism	CPS1, CTH, GLS

Table 1b, pathways downregulated by JQ1

KEGG pathways	Genes down-regulated in JQ1 treated cells
Cell cycle	CHEK2, cdc23, cdc45, ESPL1, CDK1, TGFB2, BUB1B, TTK, ORC6L, MCM3, MCM6, MCM5, CCNA2, CHEK1, WEE1, SKP2, E2F3, MCM2, E2F2, ORC1L, CDK6, cdc25C, cdc25A, CDC6, RBL2, MAD2L1, MYC
DNA replication	POLE2, MCM3, MCM6, MCM5, DNA2, RFC4, RFC5, POLA1, PRIM1, MCM2
Pyrimidine metabolism	CAD, POLE2, PNPT1, CTPS, POLR1B, POLA1, PRIM1, POLR3G, DHODH, CANT1, NME6
Systemic lupus erythematous	HIST2H2AB, HIST1H2AL, HIST1H2BL, HIST1H2AD, HIST1H2AE, H2AFY2, HIST1H2BL, HIST1H3L, HIST1H2AB, HIST1H4A, HIST1H2BM, HIST1H2BN, HHIST1H2AJ, HIST1H3J
Homologous recombination	RAD54L, BRCA2, XRCC2, EME1, RAD51, BLM
Purine metabolism	POLE2, AK2, PDE3B, PRIM1, CANT1, PFAS, ADSL, NMD6, ADCY3, PNPT1, POLA1, POLR1B, POLR3G
Progesterone mediated oocyte maturation	PDE3B, CDC25C, CDC23, CCNA2, CDC25A, ADCY3, MAD2L1, CDK1
Mismatch repair	RFC4, RFC5, MSH2, EXO1

Table 1a and 1b. pathways upregulated or downregulated by JQ1. Upregulated and downregulated genes in different pathways are listed.

Fig. 2

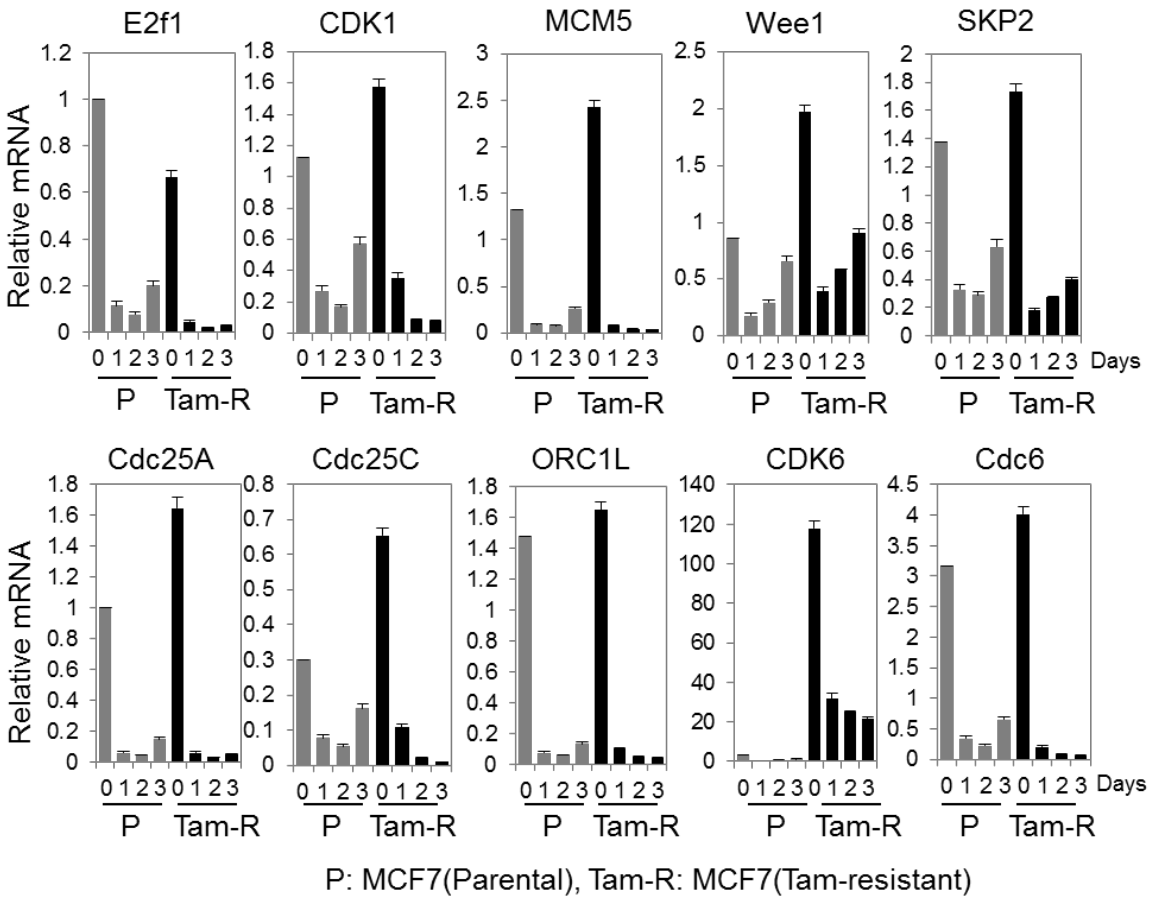


Fig. 2. Expression of cell cycle-related genes in JQ1-treated breast cancer cells. Parental and Tam-R MCF7 cells were treated with 0.2 μM of JQ1 for different days and gene expression level was determined by RT-qPCR. Error bars were shown as s.e.m.

Fig. 3

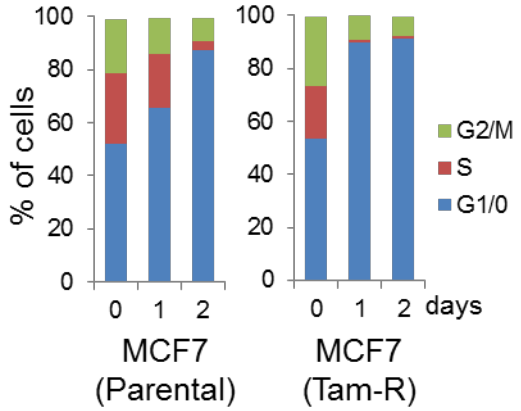


Fig. 3. Tam-R MCF7 cells are more sensitive to JQ1-induced G1 cell cycle arrest. Cells were fixed and stained with propidium iodide (PI) before being analyzed by flow cytometry.

Fig. 4

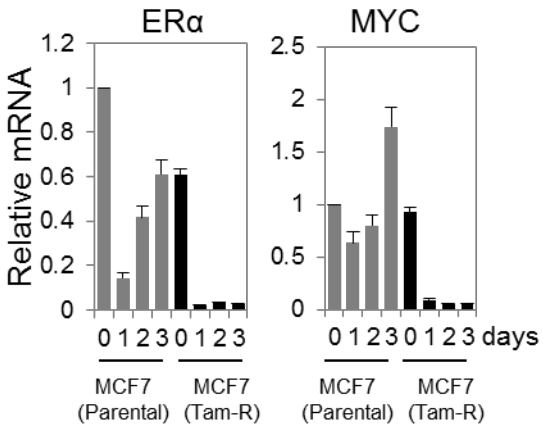


Fig. 4. JQ1 suppressed both ERα and MYC signaling pathways in Tam-R MCF7 cells. Parental and Tam-R MCF7 cells were treated with JQ1 for different days, and mRNA levels of ERα and MYC were analyzed by RT-qPCR.

Fig. 5

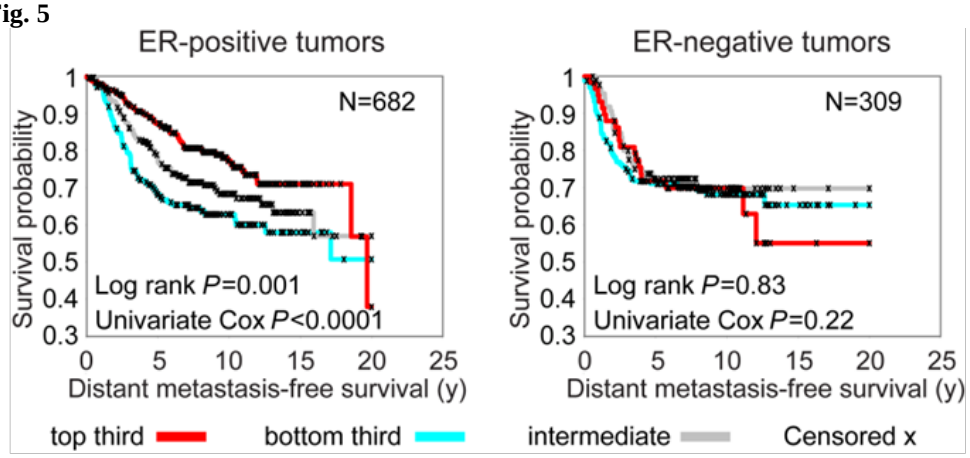


Fig. 5. Association of the gene expression signature of JQ1 treatment with breast cancer patient survival. For ER-positive and ER-negative subsets, the differences in risk between tumors, according to degree of manifestation of the JQ1 gene signature, is compared using Kaplan-Meier plots (top third, "strong manifestation"; bottom third, "weak manifestation"; middle third, "intermediate manifestation").

Fig. 6

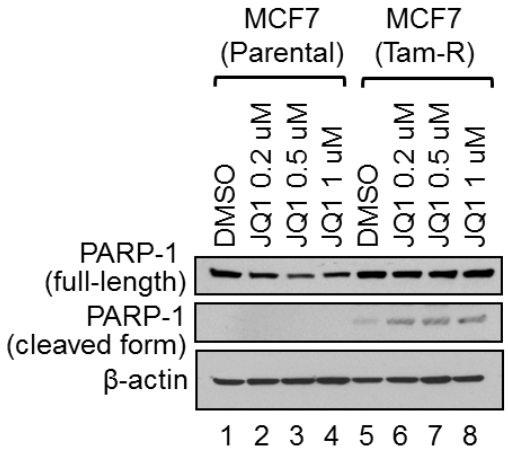


Fig. 6. JQ1 induced apoptosis in Tam-R MCF7 cells. Cells were treated with various dosages of JQ1 for 2 days before harvest for Western blot.

Fig. 7

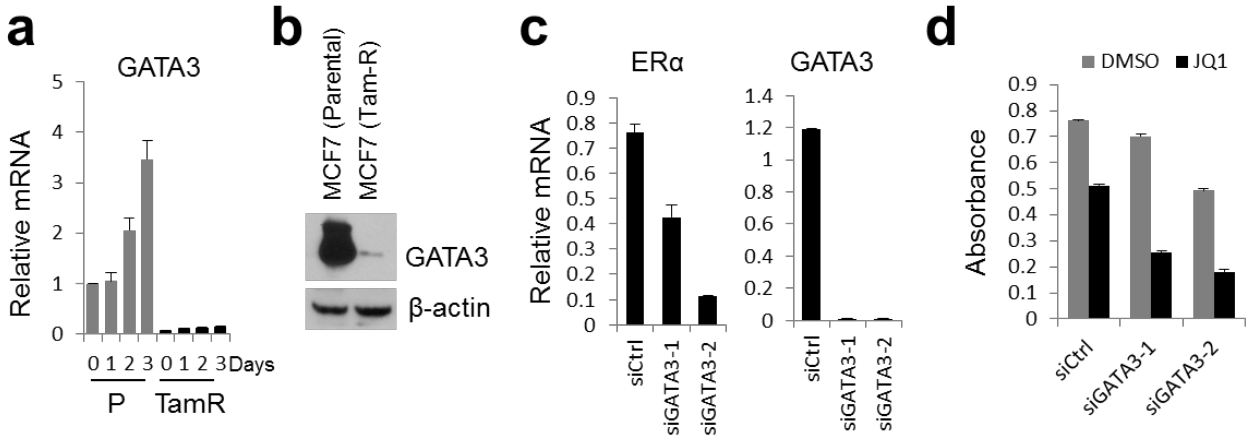


Fig. 7. (a) Expression of GATA3 in MCF7 parental and Tam-R cells after JQ1 treatment. Error bars were sown as s.e.m. (b) Comparison of GATA3 protein levels by Western blot analysis in MCF7 parental and Tam-R cells. (c) Knockdown of GATA3 by siRNA reduces ERα gene expression. Error bars were shown as s.e.m. (d) Knockdown of GATA3 by siRNA enhances JQ1 inhibition function in MCF7 parental cells. Error bars were shown as s.e.m.